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Abstract

During the past few years the Committee on Enzyme Nomenclature of the Manufacturing Section of the American Dairy Science Association has collected data on numerous enzymes in bovine milk. At least 20 enzymes have been either purified or isolated from milk or definitely identified to be native constituents of milk. However, nomenclature is not uniform; neither does there exist any uniformity among the assay procedures, substrates used, or units expressed concerning the same enzyme by various workers.

All major data concerning these enzymes have been compiled and consolidated in tabular form for convenient referral by future researchers. Also, attempts have been made to discuss the physiological as well as technological significance of these enzymes.

Besides these 20 enzymes, several workers have detected the activity of 15 other enzymes in milk. However, since only a limited amount of information is available regarding them and it has not been established that they are inherently present in bovine milk, these additional enzymes and their references have been listed only.

Introduction

The past three decades have witnessed a rapid growth in the science and application of enzymology. Also, during the same time a large number of new enzymes have been recognized and studied. Concomitantly, difficulties in terminology and nomenclature of enzymes have arisen. Early workers named identical or similar enzymes by different names because of the dissimilar isolation or assay methods. As recently as 1965, Florkin and Stotz (31) recognized confusion in nomenclature of enzymes and felt that the situation was getting out of hand. Therefore, in consultation with the International Union of Pure and Applied Chemistry, an International Commission on Enzymes was set up under the Union of Biochemistry. Similar confusion exists in the literature on milk enzymes, as indicated in the review of various milk enzymes, their role and significance (96).

The subject of the nomenclature classification and methodology of the proteins of bovine milk has been under intensive study for nearly two decades by the committee called by that name. Although during recent years notable advances have been made in the field of dairy and food enzymology, the Protein Nomenclature Committee of the American Dairy Science Association has not included the nomenclature of enzymes in their charge since enzymes fall in the category of minor proteins.

This committee on enzyme nomenclature was first appointed in 1965-66, and during the past few years it has collected data on the numerous bovine enzymes in the literature. Nomenclature is not uniform; neither does there exist any uniformity among the assay procedures, substrates used, or units expressed by various workers for the same enzyme. This committee recommends that the enzyme nomenclature proposed by the International Union of Biochemistry (IUB) be adopted by the American Dairy Science Association and that all papers published in the Journal of Dairy Science follow that nomenclature.

Most enzymes can be classified broadly according to the substrates they act upon. For example, carbohydrases act upon carbohydrates; proteases act upon proteins; lipases act upon lipids, and so forth. The classification proposed by the IUB, however, appears to be the one most widely used, according to which enzymes are divided among six major classes:

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Oxidoreductases, which catalyze oxidation or reduction reactions; Transferases, which catalyze the transfer of specific chemical moieties; Hydrolyases, which hydrolyze substrates with concomitant uptake of water molecules; Lyases, which remove or add specific chemical moieties to their substrates; Isomerases, which catalyze isomerization; and Ligases, which catalyze the synthesis or bonding together of substrate units. Each class is then divided into subclasses, each subclass divided into sub-subclasses and, finally, each sub-subclass contains several enzymes. For example, the serial classification number for lipase is 3.1.1.3. In this number the first digit 3, from the left, represents the class of hydrolases; the second digit 1 represents subclass, enzymes acting on ester bonds; the third digit 1 represents sub-subclass of carboxylic ester hydrolyases; and the final digit 3 represents the enzyme glycerol ester hydrolase, the systematic name for lipase. Most of the important milk enzymes, however, belong to the classes of oxidoreductases, such as peroxidase or catalase, and hydrolases, such as amylase, protease, phosphatase, or lipase.

Milk produced by healthy cows under normal conditions contains a variety of enzymes. In 1966 Shahani (96) reported that bovine milk contained at least 19 enzymes which had been either purified, isolated, or definitely identified. In 1971 Got (39) suggested that milk may contain as many as 55 to 60 enzymes. However, they discussed only 9 or 10 enzymes in detail and provided only references for several other enzymes.

Relatively little or no definitive information is available concerning the role or significance of enzymes in milk. In medical enzymology, the determination of enzyme activity in serum or excretions is a part of clinical diagnosis of various diseases, cell damage, or physiological aberrations (6). It has been suggested that because of damage to the cell, enzymes pass down or are released into serum or extracellular space. However, no direct or quantitative relationship has been demonstrated between the extent of cell damage and enzyme concentration. In the case of milk, catalase and Aesterase increase during udder disease and, therefore, may by physiologically important.

Also, it is believed that these enzymes are normal constituents of cells or tissues, and during the milking process concomitant with the cell rupture these enzymes are spilled into milk. A good case for such a theory could be developed on the fact that nearly 400 to 500 volumes of blood must pass through the mammary gland to produce one volume of milk. Certainly, ribonuclease of milk has been identical to pancreatic ribonuclease from where it is transported by blood to the mammary gland. Dowben and Brunner (23) suggest that all the milk enzymes originate in the secretory epithelial. On the other hand, some believe these enzymes are secreted in milk for the benefit of the young having rather underdeveloped or incomplete digestive systems.

Milk is not a homogeneous solution of enzymes. Enzymes occur in four distinct phases: (i) water soluble, (ii) associated with cream or lipids, (iii) bound to casein, and (iv) enzymes present in the microsomal particles. In their native states, these exist in an equilibrium that can be modified by chemical, mechanical, or physical means. Enzymes in bovine milk have been listed alphabetically in Table 1. The number in parentheses under each enzyme refers to the enzyme classification number as suggested by the Committee on Enzymes of the IUB (31). Below the enzyme classification numbers are the systematic or trivial names. Also included in the table is information regarding their source, isolation or purification procedure, assay procedure, substrate, and other distinguishing characteristics such as the pH and temperature optima, coenzymes, activators, inhibitors, etc. In addition, brief remarks on these enzymes follow.

Aldolase. A glycolytic enzyme, aldolase hydrolyzes fructose-1, 6-diphosphate into dihydroxyacetone phosphate and phosphoglyceric aldehyde. The publication of Polis and Shmukler (85) constitutes perhaps the first major work on this enzyme of milk.

Amylase. This enzyme catalyzes the hydrolysis of the α-1-4-D-glucosidic linkages in starch and glycogen. Although the presence of amylolytic activity in bovine milk was observed earlier, Richardson and Hankinson (88) were the first who demonstrated that milk contained both α and β-amylases, catalyzing the dextrinization and saccharification of starch, respectively. Alpha-amylase is inactivated by the pasteurization temperatures, but β-amylase is fairly heat resistant. Guy and Jenness (43) and Manolkidis (63) have concentrated α-amylase from whey and observed its pH optimum to be 7.4. Although α-amylase is considered a normal constituent of cow's milk, β-amylase is only in some milks.

Carbonic anhydrase. This enzyme reversibly catalyzes the hydration of CO_2 and dehydration of carbonic acid. Only limited work has been done on this enzyme (1, 103), and in a

TABLE 1. Enzymes in bovine milk.

Enzyme	First reported	Distribution, milk or cream	Specificity	Assay	Substrate	Optimum pH	Optimum temp (C)
Aldolase (4.1.2.13) Fructose 1-6 phosphate D-glyceraldehyde phosphatelyase	1950	Conc in cream phase	Ketose mono- and diphosphates	Measurement of phosphoglyceric aldehyde as 2,4-dinitrophenyl hydrazine derivative	Fructose, 1,6-diphosphate	Assayed at pH 7.0	35
Amylase (3.2.1.1) α-1, 4-Glucan-4-glucanohydrolase	1904	Lactoglobulin fraction of skimmilk	α-1, 4-D Glucan linkages in polysaccharides with 3 or more consecutive α-1, 4-D glucan linkages	Starch-iodine assay or loss of viscosity through hydrolysis of starch	Starch, glycogen, polysaccharides and oligosaccharides	7.4	34
β-Amylase (3.2.1.2) α-1, 4-Glucan maltohydrolase)	1936	Lactoglobulin fraction of skimmilk	α-1, 4-Glucan linkages in polysaccharides to remove successive maltose units from nonreducing end	Assay for maltose, or dinitrosalicylic acids assay of sugar	Starch, glycogen, poly- and oligosaccharides	NR	NR
Carbonic anhydrase (4.2.1.1) Carbonate hydrolase)	1953	NR	Carbonic acid-CO_2 interconversion	CO_2 and carbonic acid	Carbonic acid	NR	NR
Catalase (1.11.1.6) Hydrogen peroxide oxidoreductase)	1907	Milk microsomes, casein, or lactoglobulin fraction	H_2O_2	Assay of O_2 formed from H_2O_2	H_2O_2, Ethanol reported as hydrogen acceptor	7.0	NR 2a
Cytochrome c reductase (1.6.99.3) Reduced NAD: cytochrome C oxidoreductase)	1954	Fat globules	NR	Reduction of substrate spectrophotometrically	NADH + acceptor	7.3	18
Diphorase (1.6.4.3) Lipoamide dehydrogenase) Reduced NAD: oxidoreductase)	1954	Fat globules	NR	Reduction of 2,6 dichloro phenol indophenol spectrophotometrically	NADH + lipoamide	7.3	18
Esterase (3.1.1.2) Arylesterase)	1901 1959	NR	Aryl esters not tributyrin	Manometric titrimetric	Phenyl acetate	8.0	37
Esterase (3.1.1.1) Carboxylesterase)	1959	Probably aqueous phase	n-Methyl esters of fatty acids triglycerides	Manometric in presence of 10^{-6}M TEPP	Tributyrin	As above	As above
Esterase (3.1.1.8) Cholinesterase)	1959	NR	Choline esters tributyrin	Manometric in presence of 10^{-6}M TEPP by difference	Phenyl propionate	As above	As above 3a
Lactose synthetase (2.4.1.22) UDP galactose: glucose, 1-galactosyl transferase)	1964	Aqueous phase	UDP-galactose and α-D-glucose	Uptake of C^{14} into lactose. UDP assay	UDPG galactose + α-D-glucose	7.2	37
Lipase (3.1.1.3) Glycerol-ester hydrolase)	1922	Skimmilk	Glycerol-esters in emulsion	Detm. of FFA by extraction, chromatography, or pH stat	Milk fat, tributyrin, and glycerides	9.0 to 9.2	37
Lysozyme (3.2.1.17) Mucopeptide acetylneuraminyl hydrolase)	1932	Whey	β-1, 4 linkage between N-acetyl glucosamine and n-acetylmuramic acid		Cell walls or M. lysodeikticus cells	7.9	37
Phosphoprotein phosphatase (3.1.3.16) Formerly acid phosphatase phosphoprotein phosphohydrolase)	1945	Cream:skimmilk 2:1	Phosphate esters with phenol OH group, ADP, ATP, casein, organic and inorganic pyrophosphate	Detm. of p-nitrophenol or phosphate	Phosphate ester	4.0 to 5.5	14 to 50
Phosphatase alkaline (3.1.3.1)	1933	Cream:skimmilk 7:4	Phosphate monoesters, serine-P, β-glycerol-P AMP	Release of phenol from phenyl phosphate or phosphate from β-glycerophosphate	Phosphate ester	9.8	37

TABLE 1.

Purification achieved	Coenzyme or cofactor	Activators	Inhibitors	Physicochemical characteristics	Remarks
Removal of casein and ppt with ammonium sulfate conc 50×	NR	NR	Acids	Heat-labile, unstable in milk at 37 C.	In milk at about same conc as in blood
Adsorbed on starch from lactoglobulin B fraction of whey and desorbed with calcium, conc 30×	Ca^{++} and Cl^-	NR	NR	Inactivated at 55 C for 30 min	Predominant amylase in milk
Conc from lactoglobulin	NR	$CaCl_2$	NR	Withstands 65 C for 30 min	Accepted as a constituent of some milks
NR	Zinc	NR	NR	...	Probably of blood origin
NR	Heme	NR	Acids	Inactivated by H_2O_2 above 10 C, heat-labile	Conc increases with leukocytes in milk
Activity in milk microsomes. Conc by churning and centrifugation of buttermilk	NR	NR	NR	NR	2b Associated with milk microsomes. Tendency to be adsorbed onto fat globules. Similar to those found in mammary tissue
Activity in milk microsomes. Conc by churning and centrifugation of buttermilk	NR	NR	NR	NR	
Activity detected	NR	NR	Resistant to organo phosphates	NR	Similar to serum enzyme. Correlated with mastitis. Hydrolyzes some organophosphates
Activity detected, lipase activity conc by adsorption on $Mg(OH)_2$ 10-20×	NR	NR	Parathion	NR	Some hydrolysis of simple esters may be due to milk proteins. Conc B-esterase acts like lipase on emulsified glycerides
Activity conc 400× by salt fractionation and ion exchange chromatography	NR	NR	Parathion, MDFP, or TEPP	NR	High conc in colostrum
Centrifugation 30× $(NH_4)_2SO_4$ pption column chromatography separated into two fractions A & B. B crystallized		0.04 M Mn	Higher conc Mn, Mg, Tri-PO_4	UDP, ppi	3b Found in milk of cow, sheep, goat and human. Fraction B very stable. Neither active alone. Fraction B identical with α-lactalbumin.
From clarifier sediment extraction and chromatography 88-fold. Dissociation from casein by dimethyl formamide	NR	Homogenization, shaking, temp manipulation	Heavy metals	Heat-labile	Milk proteins protect it.
Adsorption on Amberlite, precipitation and gel filtration 36,000-fold	NR	NaCl	NR	Heat stable at low pH. Imidazole and indole derivatives	...
Adsorption and chromatography. Starch gel electrophoresis 40,000×	NR	Ascorbic acid	F	Relatively heat stable. Basic protein	...
From buttermilk by butanol dissociation adsorption and precipitation 5,660-fold. From skim 5,000-fold.	Mg	NR	Borate, I, cystein metal complexing agents.	Inactivated at 67 C for 10 min. Stable to organic solvents	Does not hydrolyze diesters, pyrophosphate or polyphosphate.

TABLE 1.

Enzyme	First reported	Distribution, milk or cream	Specificity	Assay	Substrate	Optimum pH	Optimum temp (C)
Peroxidase, (1.11.1.7) hydrogen-peroxide oxidoreductase	1881	Whey	H_2O_2 and some organic peroxides	Colorimetric	H_2O_2 + reducible agent	6.0 to 7.0	20
Protease (3.4.4.–) (Peptidohydrolase)	1897	Casein	NR	Colorimetric detm. for a.a. measuring COOH or NH_2 groups	Tryptic and/or chymotryptic specificity	8.0 6.5	4a 37
Rhodanese (2.8.1.1) (Thiosulfate: sulfurtransferase)	1933	NR	NR	Colorimetric	CN	NR	NR
Ribonuclease (2.7.7.16) (Polyribonucleotide 2-oligonucleotide transferase)	1963	Whey	Pyrimidine cyclic nucleotides, polyuridine	Spectrophotometric	RNA	7.5	37
Salolase	1907	NR	Phenyl salicylate	NR	Salicylate	NR	NR
Xanthine oxidase (1.2.3.2) (Xanthine-oductase)	1902	Fat globules	NR	M.B. as H acceptor; manometric. Xanthine as substrate. Triphenyl tetrazolium chloride as H acceptor. Vanillin to vanillic acid	Aldehydes, Oxypurines, Pterin, DPN, and Xanthine	6 to 9	37

recent investigation Kitchen et al. (59) could not detect any carbonic anhydrase activity in any of the samples of milk they tested.

Catalase. This is one of the initial enzymes reported in milk (104), and since its discovery a considerable amount of work has been done on its isolation and development of an assay method for its detection (62, 76). It catalyzes the decomposition of hydrogen peroxide into water and oxygen. Since the concentration of this enzyme varies directly with the concentration of bacterial and leucocyte counts in milk, its occurrence in normal milk was questioned. However, the presence of this enzyme in aseptically drawn milk from healthy animals has established that it is a native constituent of normal milk. More recently, Kitchen et al. (59), while studying milk enzymes, observed a higher specific activity of catalase, aldolase, xanthine oxidase, and phosphatase in the fat fraction. Also, the catalase content of milk has been used as a means of detecting mastitic milk.

Cytochrome C reductase. Activity of this enzyme was concentrated in milk microsomes isolated by centrifugation of buttermilk. Its activity is assayed by measuring spectrophotometrically the rate of reduction of cytochrome C. The milk microsomes are similar to those in mammary tissue and show a marked tendency to adsorb onto milk fat globules (71).

Diaphorase. This enzyme catalyzes the dehydrogenation of lipoamide in the presence of a hydrogen acceptor, and its activity is measured by following the reduction of 2, 6-dichlorophenol indophenol. In milk the enzyme appears to be associated with microsomes (76).

Esterase. As early as 1901 monobutyrin was hydrolyzed by milk, indicating that milk possesses an esterase activity (104). As many as three esterases have been found in milk. The term esterase embraces a variety of enzymes which catalyze the hydrolysis of esters (54, 104). Although lipase is also an esterase, its activity generally is regarded to be confined to glycerol esters.

Many workers have noted that milk would hydrolyze simple esters. The validity of many of these reports, however, is doubtful because some of the substrates were impure and be-

TABLE 1.

Purification achieved	Coenzyme or cofactor	Activators	Inhibitors	Physicochemical characteristics	Remarks
Chromatography of whey or skim	Bound hematin	NR	CN, F	Absorption ratio at 480:412 mu 0.9 mol wt 82,000	Crystallized
Wash acid casein, pH 4.0, ppt supernatant with Am SO_4, DEAE chromatography 180-fold	NR	NR	1% alcohol and 15% NaCl in acid medium	Heat labile at 75-80 C, and in acid solution destroyed at 72 C in 10 min, diisopropylfluorophosphate, soy bean trypsin inhibitor	4b HTST temp. may reactivate the enzyme. Associated with casein & casein itself is the substrate. Hydrolysis rate for caseins is $\beta > \alpha_s > K$
NR	NR	NR	NR	NR	It has detoxication effect
Chromatography on IRC-50	NR	EDTA, NaF	Zn, Cu, Hg	Mol wt 13,600; pI 7.8; basic protein. Resistant to heat at 90 C, pH 3.5; it consists of five isozymes containing varying amounts of carbohydrate	Major milk RNase identical to pancreatic RNase. RNase may affect RNA of fat globule membrane and, hence, may influence the emulsion stability
NR	NR	NR	NR	...	Present in mammary gland
Crystallized	Might be flavin adenine dinucleotide	NR	Cyanide arsenite H_2O_2 p-Chloromercuribenzoate	Mol wt 300-400,000 pI 5.3-5.4. The enzyme consists of protein, flavin, Mo, and Fe. The molar ratio of Fe to Mo to flavin in crys. prep., 8:1.4:2	Occurs in microsomes which contain protein and lipids. Attached to the fat globules. May be found in plasma depending upon treatment of milk; ave. content in milk 160 mg/l

cause of the recent finding by Downey and Andrews (24) that about 70% of the esterase activity in milk toward *p*-nitrophenylacetate was due to nonenzyme proteins. Approximately 20% of the activity was catalytic.

The most comprehensive work on milk esterases has been accomplished by Forster and co-workers (32-34, 64-66, 70, 71). Utilizing the inhibitory effects of various organophosphates, they obtained evidence for three esterases in milk, A, B, and C (33). A-Esterase (aryl esterase) is a typical aromatic esterase which hydrolyzes phenyl acetate at a higher rate than phenyl butyrate. Aliphatic esters normally are not attacked. It is resistant to both organophosphate compounds and physostigmine (eserine). B-Esterase (carboxy or glyceryl ester hydrolase) hydrolyzes aliphatic and aromatic esters but not choline esters. It is sensitive to organophosphates but not to eserine. C-Esterase (cholinesterase) splits choline esters more rapidly than aliphatic or aromatic esters and is sensitive to organophosphates and eserine (34).

Assays were manometric at pH 8.0 and 37 C in the presence of various inhibitors. Thus, A-Esterase activity was determined with phenyl acetate as the substrate and 10^{-2} M parathion which inhibited B and C Esterases. Tributyrin was the substrate for B-Esterase. This compound was not hydrolyzed by A-Esterase. C-Esterase hydrolysis of tributyrin was inhibited by 10^{-6} M tetraethyl-pyrophosphate (TEPP). C-Esterase activity is determined with phenyl propionate as the substrate and 10^{-6} M TEPP as the inhibitor. The part of the hydrolysis inhibited by 10^{-6} M TEPP was ascribed to C-Esterase.

Recently, Kitchen (58) also observed that the major type of esterase in skimmilk was an aryl esterase; choline-esterase was in smaller amount.

A and C-Esterase activity is high in colostrum, and the former was high in mastitic milk. A-Esterase activity of milk appears to be related to mastitis (54). The pH and temperature optima were 7.1 and 37 C. In order to utilize fully the A-Esterase assay as a screening test for mastitis, it was necessary to establish the levels in bovine sera (65). It was sug-

gested that A-Esterase in milk is the result of transudation from sera, which contain much more enzyme than normal milk. Milk from cows with clinically induced mastitis showed increased A-Esterase activity (66). A-Esterase hydrolyzed several of the organophosphorus compounds used as inhibitors, notably diisopropylfluorophosphate, TEPP, and N,N′, diisopropylphosphordiamidofluoridate (32).

C-Esterase activity was noted in colostrum, where both butyryl and propionyl choline-p-toluene sulfonate were hydrolyzed more rapidly than acetylcholine. Tributyrin was hydrolyzed at an appreciable rate. Activity was eliminated completely by 10^{-5} M eserine. The enzyme has been purified over 400-fold by a combination of salt fractionation and ion-exchange chromatography (71).

Contrary to the statement by Downey and Andrews (24), Forster et al. (34) did not use *p*-nitrophenylacetate as a substrate in their work. This was investigated, but nonenzymatic hydrolysis was so great that phenyl acetate was used instead. This compound showed practically no nonenzymatic hydrolysis. Forster et al. (34) found that milk definitely contains heat-labile substances which hydrolyze simple esters such as methyl and ethyl butyrate. They did not attempt to determine which of the three esterases was most responsible.

B-Esterase also has been concentrated (70) and Jensen (54) observed that it appears to behave, as far as specificity was concerned, identically to the lipase in whole raw milk and to the isolated lipase of Chandan and Shahani (13). This esterase definitely contains glycerol hydrolase activity.

Lactase or β-galactosidase. This enzyme catalyzes the hydrolysis of β-D-galactoside, producing glucose and D-galactose. Although this enzyme has been listed as being in milk, its presence has not been substantiated fully. If present, milk would contain more glucose and galactose than it does, and their content would increase with time if bacterial growth was controlled (103, 104). Many species of bacteria produce β-galactosidase.

Lactose synthetase. As the name indicates, it catalyzes the synthesis of lactose from UDP-galactose and α-D-glucose. This enzyme originates from the microsomes of mammary cells of lactating animals. Lactose synthetase was isolated from milk by Babad and Hassid (4). The material was obtained by centrifugation, $(NH_4)_2SO_4$ precipitation, and dialysis. The enzyme was assayed by determining the amount of C^{14} incorporated into lactose when incubated with UDP-D galactose-1-^{14}C and D-glucose. The optimum conditions for the assay were described as pH 7.5 and 37 C. Lactose synthesis did not occur if α-D-glucose-1-phosphate was substituted for D-glucose. Some oligosaccharides probably were synthesized but were not investigated further. Manganese (Mn^{++}) was stimulatory at .04 M but was inhibitory at higher concentrations.

Brodbeck and Ebner (12) similarly isolated lactose synthetase (30-fold) and then on a column of Bio Gel P 30 separated it into two fractions, designated A and B. No synthetic activity was found when either fraction was assayed separately. Fractions A and B were also obtained from the skimmilk of the sheep, goat, and human. Fraction A is heat labile whereas fraction B withstands boiling for 20 min at pH 7.4 without loss of activity. Fraction B retained full activity after precipitation with 10% trichloroacetic acid. Microsomal lactose synthetase from bovine mammary tissue was solubilized by sonic oscillation. The addition of soluble fraction B to these extracts greatly stimulated synthetase activity, suggesting that fraction B is limiting in microsomes.

Brodbeck et al. (11) further purified fraction B by chromatography on DEAE-cellulose and crystallization. The crystals were identified as α-lactalbumin by the following criteria: substitution in the enzymic rate assays, spectra, immunological titrations, amino acid composition, mobility on starch gel electrophoresis, molecular weight, and cochromatography on DEAE-cellulose and Sephadex G-100. Ebner and Schanbacher (27) studied the galactosyl acceptor specificity of the A protein of lactose synthetase. They reported that both α-lactalbumin and galactosyl transferase were necessary for lactose synthetase activity. Alpha-lactalbumin inhibited the transfer of galactose and also modified galactose transferase in a manner that the enzyme could utilize glucose as a substrate. The acceptor specificities of galactose transferase suggested that lactose and certain glycoprotein synthesis would be by the same enzyme. Additional information on this enzyme is available in recent reviews of Ebner (26) and Rose et al. (92).

Lipase. Lipase catalyzes the hydrolysis of glycerol-esters (fats and oils) in emulsion. Although its presence in milk was established conclusively in 1922 (87), as early as 1904 Rogers (91) observed an increase in the acidity of canned butter. Among the milk enzymes, lipase is perhaps the most extensively studied enzyme. During recent years several workers

(24, 35, 37, 44, 94) have isolated many lipases, each differing from the other in many respects. By high speed centrifugation Harper et al. (44) obtained two lipases—one in the supernatant with a pH optimum of 7.0 and the other in the sediment with a pH optimum of 8.6. On the other hand. Gaffney et al. (37) and Downey and Andrews (24) obtained several casein fractions with variable lipase or tributyrinase activity. However, none of the fractions was homogeneous.

In 1963, Chandan and Shahani (13) purified milk lipase from clarifier slime, which was ultracentrifugally as well as electrophoretically pure and homogeneous. Using a modification of the Chandan and Shahani technique, Richter and Randolph (90) also obtained a pure lipase from clarifier slime, which possessed essentially the same properties as those reported earlier (14). Recently, Rout (93) reported that lipase chromatographs with casein on Sephadex G-100, with water as the eluant. However, with phosphate buffer the lipase fraction spreads, yielding a broad band or several peaks.

The major milk lipase is associated with casein or with an individual component of the casein complex. The isolated milk lipase appears to be a true lipase in that it hydrolyzes only esters of glycerol in a two-phase heterogeneous emulsified system and does not hydrolyze simple esters in solution (14, 46, 82). It possesses a single pH optimum of 9.0 and a temperature optimum of 37 C. It is unstable and highly sensitive toward light, heat, and several reagents. Various milk constituents have a profound effect upon its activity—salts and caseins being inhibitory, lactose having no effect, and albumins and globulins being stimulatory. Therefore, the observed lipase activity in a complete milk system may be the net result of the inhibitory and stimulatory action of various milk constituents upon the lipase (98). With a molecular weight of 7,000 to 8,000, it is probably the smallest enzyme ever isolated (18). Rout (93) also observed that one of the two lipase fractions isolated had a molecular weight of less than 10,000. A considerable amount of work on lipase specificity has been done by Jensen (54). Additional information on lipase is available in a review by Chandan and Shahani (15).

Lysozyme. It catalyzes the hydrolysis of β (1-4) linkage between N-acetyl glucosamine and N-acetylmuramic acid present in polymer form in the bacterial cell wall (51). In 1932 Fleming (30) observed lysozyme activity in cow's milk. Also, the milk of donkey, mare, cat, goat, and rabbit definitely contains lysozyme but only trace amounts or none has been in the milk of sheep, sow, or guinea pig (17, 96). Jenness (52) reports high concentrations of lysozyme in the milk of primates and carnivores and little, if any, in the milk of rodents and artiodactyls (split-hoofed animals). Bovine milk contains 13 μg of lysozyme per 100 ml while human milk contains 39 mg/100 ml (83).

Lysozyme lyses bacteria and, therefore, might be associated with the natural antibacterial factors in fresh milk. It appears to be located in the skimmilk or whey portion of milk, and Chandan et al. (16) isolated it in a pure form by absorption from acid whey onto IRC 50, precipitation, and gel filtration. It is remarkably stable to heat, particularly at acid pHs. It is a relatively small protein, with a molecular weight of approximately 18,000 which is slightly higher than that of the egg white lysozyme (29). Recently, considerable work on this enzyme has been done (28, 36, 81, 97) concerning its assay, physicochemical properties, amino acid composition, peptide fingerprinting, immunological characteristics, and the role of various amino acids in its activity.

Peroxidase. It catalyzes the decomposition of hydrogen peroxide in the presence of an oxidiable compound or hydrogen donor. Milk peroxidase, often referred to as lactoperoxidase, is perhaps the first enzyme reported in milk (2). Hydrogen peroxide or such reducible agents as pyrogallol or guaiacol are used as substrates in its assay (64, 99, 100). It has been isolated from milk and crystallized (72, 73, 86). It appears to be associated with the albumin or whey protein components of milk. It constitutes about 1% of the total serum protein content, being the highest in concentration in relation to other enzymes. Also, compared to other enzymes it is fairly heat stable (53).

Phosphatases. This class of enzyme hydrolyzes phosphoric acid esters. A large number of phosphatases exist in nature such as phosphomonoesterases, phosphodiesterases, phosphorylases, pyrophosphatases, and phosphoprotein-phosphatases. Milk contains several phosphatases, but most of the work on phosphatases in milk has been done on two types: alkaline phosphatase, a phosphomoesterase with a pH optimum of 9.6; and the acid phosphatase, which is not a phosphomonoesterase and has a pH optimum of 4.0 (53).

In the literature the enzyme referred to as

acid phosphatase (49) appears to be named wrongly, and because of its substrate specificity it should be classified appropriately as phosphoprotein-phosphatase (PPPase). Mullen (80) showed that milk PPPase hydrolyzed phenolphosphate with a pH optimum of 4.0. However, he reported that the enzyme was slow in hydrolyzing other phosphate esters, such as α- and β-glycerolphosphates. Bingham and Zittle (9) purified the enzyme over 40,000-fold and studied its properties. The PPPase acts on aromatic phosphates, casein, and pyrophosphates (both organic and inorganic). It did not act on adenosinemonophosphate, serine phosphate, or glycerol phosphate —typical phosphomonoesterase substrates. Ascorbic acid will triple the enzyme activity. Bingham and Zittle were the first to point out that the PPPase of skimmilk resembles the PPPase of bovine spleen. Both enzymes are active with the same type of substrates, are activated by ascorbic acid, and are highly basic proteins. Certain differences are evident, however, between the enzymes: (i) the milk enzyme showed little activation with thioglycolate, cysteine, mercaptoethanol, or N-ethylmaleimide, all of which activate spleen PPPase; (ii) ascorbic acid inactivates spleen PPPase when preincubated with the enzyme, but the milk enzyme is not inactivated; and (iii) the milk enzyme has relatively low activity against casein compared to other substrates.

Bingham et al. (7) showed that while PPPase was associated with the soluble fraction of skimmilk, the enzyme in cream was insoluble and probably associated with the lipoprotein complex of the fat globule membrane. Although the milk enzyme and cream enzyme are similar in many respects, there is not enough information to classify the cream enzyme as either PPPase or acid phosphatase.

Alkaline phosphatase was first observed in milk in 1933 (40). Its concentration in milk is highly variable, but the enzymes appear to be associated with the microsomal particulates like PPPase. Extensive work has been done by Morton (74-79), Zittle and associates (108-110), and Kitchen et al. (59) on its purification and characterization. It has been purified from both buttermilk and skimmilk, achieving a better than 5,000-fold purification. It is activated by Mg^{2+} and Mn^{2+} (77, 78). It is relatively stable to organic solvents but is inhibited by zinc, iodine, cysteine, and metal complexing agents.

Pasteurization inactivates alkaline phosphatase and, therefore, phosphatase activity in milk is used as an indicator for proper pasteurization or postpasteurization contamination. However, under certain conditions, milk and milk products subjected to ultra high pasteurization treatments show reactivation of phosphatase (89).

Protease. Although the first report of protease in milk was in 1897 (5), it was not until 1960 that Harper et al. (45) reported that proteolytic activity of milk was due to an inherent protease and not to bacteria. Warner and Polis (102) found that the most proteolytic activity of milk is precipitated with casein when milk is acidified. Zittle (107) extended this method by washing acid curd with water acidified to pH 4.0 and ammonium sulfate precipitation of the solubilized protease. Yamauchi et al. (106) were able to achieve 70-fold purification over the unfractionated protease and examined some of the properties of this enriched fraction. Recently, Dulley (25) purified milk protease by chromatography and ammonium sulfate fractionation and observed that it had a wide pH optimum of 6.5 to 9.0. However, Kaminogawa et al. (55) observed that protease had a sharp pH optimum at 8.0 with an accompanying shoulder at 6.5, showed tryptic and chymotryptic substrate specificity, and the most effective inhibitor was diisopropylfluorophosphate. They also found that when the protein was in a more purified state, it could be destroyed by heating to 80 C for 10 min; however, when in aseptically canned milk, considerable proteolysis of the casein occurred after storage for 30 days at 30 C. Several investigators (19, 55) have found that β-casein is most susceptible to proteolysis followed by α_s- and κ-casein. Chen and Ledford (19) achieved 180-fold purification, and this preparation demonstrated mostly trypsin-like activity.

Rhodonase. Also spelled Rhodanase or Rhodanese, this enzyme catalyzes the conversion of cyanide into thiocyanate (103). It was first reported in bovine milk in 1945 (20), and the milk of goat and sheep reportedly contains higher rhodonase than bovine milk (1).

Ribonuclease. Bovine milk contains 25 mg/L of this enzyme which catalyzes the hydrolysis of ribonucleic acid. Like the ribonuclease A from bovine pancreas, milk ribonuclease is also a basic, low molecular weight protein. It exists primarily in the whey, and Bingham and Zittle (8, 10) isolated two ribonucleases, A and B, from acid whey. Using ammonium sulfate precipitation and adsorption on Amberlite IRC-50, they isolated the enzyme in a pure

form. More recently, the bovine milk ribonuclease was crystallized (41).

On the basis of such characteristics as the amino acid composition, electrophoretic mobility, immunological characteristics, and specificity, ribonuclease A of milk appears to be similar to pancreatic ribonuclease A (10, 50). Hydrolysis of RNA by this enzyme produces 3′-nucleotides with pyrimidine nucleotides preferentially released, but the hydrolysis stops at 31% reaction leaving the core (50). The 2′, 3′-cyclic nucleotides of cytidine and uridine also are hydrolyzed and polyuracil is hydrolyzed completely to single units. Also, bovine milk ribonuclease closely resembles human milk ribonuclease (21) in several characteristics.

Salolase. It catalyzes the hydrolysis of phenyl salicylate, and Vandevelde (101) appears to be the only one who has observed it in milk. It also has been detected in bovine mammary glands. Salolase activity may be associated with A-esterase activity in that both enzymes reportedly hydrolyze phenyl esters.

Xanthine oxidase. It is a rather nonspecific enzyme, since it catalyzes the oxidation of purines, pyrimidines, and aldehydes. Xanthine is oxidized to uric acid and aldehydes to acids (2, 53, 103). The term dehydrogenase is often used for this enzyme. It was first observed in milk in 1902 by Schardinger (95) and, therefore, it often is referred to as the Schardinger's enzyme. Morton (76) reported the presence of this enzyme in microsomal particles. In 1955 Avis et al. (3) crystallized the enzyme.

Xanthine oxidase is a metallo-protein containing molybdenum and iron and is a very high molecular weight protein—about 300,000. It is moderately stable to heat and, in contrast to other enzymes, its activity in milk increases with several heat treatments, homogenization, and by protease and lipase action (96).

Other enzymes. Besides the above enzymes, several researchers have detected the activity of many additional enzymes in bovine milk. However, only a limited amount of work has been reported on those enzymes. Also, there may be some question as to whether they are inherently in bovine milk or are of microbial origin. Therefore, these additional enzymes are listed only with appropriate references: β-Acetylglucosaminase (67), alanineamino transferase (47), aspartate amino transferase (47), ATPase (22, 48, 84), diamine oxidase (105), flavin-mononucleotide (FMN) adenylyltransferase (38), glucose-6-phosphatase (22), glucose phosphate isomerase (47), β-glucuronidase (56), inorganic pyrophosphatase (48), lactate dehydrogenase (60), lysylarylamidase (68), Malate dehydrogenase (60), α-mannosidase (69), 5′-nucleotidase (84), nucleotide pyrophosphatase (84), phosphodiesterase (42), riboflavin kinase (38), sulfhydryl oxidase (57), and thiol oxidase (42).

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